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Human neoplasms elicit multiple specific immune responses in the autologous host

(human tumor antigens/antibodies)

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ABSTRACT **Expression of cDNA libraries from human melanoma, renal cancer, astrocytoma, and Hodgkin disease in *Escherichia coli* and screening for clones reactive with high-titer IgG antibodies in autologous patient serum lead to the discovery of at least four antigens with a restricted expression pattern in each tumor. Besides antigens known to elicit T-cell responses, such as MAGE-1 and tyrosinase, numerous additional antigens that were overexpressed or specifically expressed in tumors of the same type were identified. Sequence analyses suggest that many of these molecules, besides being the target of a specific immune response, might be of relevance for tumor growth. Antibodies to a given antigen were usually confined to patients with the same tumor type. The unexpected frequency of human tumor antigens, which can be readily defined at the molecular level by the serological analysis of autologous tumor cDNA expression cloning, indicates that human neoplasms elicit multiple specific immune responses in the autologous host and provides diagnostic and therapeutic approaches to human cancer.**

A prerequisite for the successful application of recombinant tumor vaccines and other immunotherapeutic interventions in cancer patients is the recognition by the immune system of tumor-specific and tumor-associated antigens (i.e., of molecules that are overexpressed or specifically expressed in the tumor cells). Despite the extensive efforts that have been made in recent years to identify such tumor antigens in human neoplasms, to date only few have been defined at the molecular level. While B-cell responses (antibodies) have been demonstrated in cancer patients when searching for antibodies against predefined molecules expressed by human tumors, such as p53 (1) or the oncogene-coded HER-2/neu protein (2), both new and known molecules were identified as tumor antigens by the analysis of the T-cell repertoire against tumors by using two strategies: first, the genetic approach established by Boon and colleagues (3), which makes use of antigen-loss tumor cell variants and cytotoxic $CD8^+$ anti-tumor T-cell clones (cytotoxic T lymphocytes), and second, the biochemical strategy (4), which is based on the acid elution of antigenic peptides bound to major histocompatibility complex class I molecules from tumor cells (5, 6). Using these approaches, several additional human tumor antigens have been defined at the molecular level, most notably in malignant melanoma (7–11). For many human tumors other than melanoma, tumor antigens have yet to be defined at the molecular level. Whether and to which extent the *in vitro* anti-tumor mechanisms of T cells that have been used to detect human tumor antigens are also operative *in vivo* remains to be proved. Moreover, little is known about the frequency of antigens expressed by a given tumor that elicit immune responses in the tumor-bearing patient. To address these questions, we modified the strategy of autologous typing (12) to allow for an unbiased search and the direct molecular definition of immunogenic tumor proteins based on their reaction with autologous patient sera. By applying this strategy of "serological identification of antigens by recombinant expression cloning" (SEREX) to four unselected human tumors of different origin, we discovered an unexpected frequency of tumor antigens that elicit specific immune responses in the autologous host.

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MATERIALS AND METHODS

Construction of cDNA Expression Libraries. The study had been approved of by the local ethical review board (Ethikkommission der Ärztekammer des Saarlandes). Recombinant DNA work was done with the official permission and according to the rules of the state government of Saarland. Total RNA was isolated from fresh tumor biopsies. Poly(A)$^+$ RNA was prepared with a mRNA isolation kit (Stratagene). cDNA expression libraries were constructed by starting with 5–8 μg of poly(A)$^+$ RNA. First-strand synthesis was performed using an oligo(dT) primer with an internal *Xho* I site and 5-methyl-CTP. cDNA was ligated to *Eco*RI adaptors and digested with *Xho* I. cDNA fragments were cloned directionally into the bacteriophage expression vector λZAPII (Stratagene), packaged into phage particles, and used to transfect *Escherichia coli*, resulting in at least 1.5×10^6 primary recombinants in each library.

Immunoscreening of Transfectants. Immunoscreening for the detection of reactive clones in each library was performed with autologous serum. *E. coli* transfected with recombinant, λZAPII phages were plated onto Luria–Bertani agar plates. Expression of recombinant proteins was induced with isopropyl β-D-thiogalactoside. Plates were incubated at 37°C until plaques were visible and then blotted onto nitrocellulose membranes. The membranes were blocked with 5% (wt/vol) low-fat milk in Tris-buffered saline and incubated with a 1:1000 dilution of the patient's serum, which had been preabsorbed with transfected *E. coli*. Serum antibodies binding to recombinant proteins expressed in lytic plaques were detected by incubation with an alkaline phosphatase-conjugated goat anti-human IgG and visualization by staining with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Sequence Analysis of Identified Antigens. Positive clones were subcloned to monoclonality and submitted to *in vivo* excision (13) of pBluescript phagemids (Stratagene). The nucleotide sequence of cDNA inserts was determined using a

Abbreviations: SEREX, serological identification of antigens by recombinant expression cloning; EMBL, European Molecular Biology Laboratory; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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T7 sequencing kit (United States Biochemical). Sequence alignments were performed with DNASIS (Pharmacia) and BLAST software on European Molecular Biology Laboratory (EMBL) and GenBank data bases (release 4/95). cDNAs not representing the whole open reading frame were submitted to a modified rapid amplification of cDNA ends protocol (14) to obtain full-length cDNAs.

Detection of Antigen-Specific Antibodies in Allogeneic Sera. Phages from positive clones were mixed with nonreactive phages of the cDNA library as internal negative controls at a ratio of 1:10 and used to transfect bacteria. IgG antibodies in 1:200 diluted *E. coli*-absorbed sera from allogeneic patients and healthy controls were detected with the immunoscreening assay described above.

Northern Blot Analysis. Northern blots were performed with RNA from tumors and normal tissues extracted by the guanidium isothiocyanate/phenol/chloroform method (15). Integrity of RNA was checked by electrophoresis in formalin/Mops gels. Gels containing 40 μg of RNA per lane were blotted onto nylon membranes. Hybridization of the Northern blots with specific ^{32}P-labeled probes was performed at 42°C with formamide. The filters were washed at 65°C with 1× standard saline citrate and 0.1% SDS and exposed for 16 hr. After exposure, the filters were stripped and rehybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Expression ratios were determined by densitometry of autoradiographs with an XRS scanner and whole band analyzer software (BioImage, Ann Arbor, MI).

Sequence Comparison with cDNAs Obtained from Normal Tissues. Sequence comparison of the identified cDNAs was performed with their counterpart obtained from normal tissue. Briefly, 10 μg of oligo(dT)-primed total RNA was reverse-transcribed with SuperScript reverse transcriptase (GIBCO/BRL). One-tenth of the reaction mixture was used for PCR amplification of specific products with oligonucleotides flanking the open reading frames of identified cDNAs. Amplification reactions were performed for 30 cycles with native *Pfu* DNA polymerase (Stratagene) with an additional incubation with *Taq* DNA polymerase (GIBCO/BRL) for 10 min at 72°C. PCR products were ligated into a TA Cloning vector (Invitrogen) and sequenced.

RESULTS

Prokaryotic Expression and Detection of Tumor Antigens by Autologous Serum. cDNA libraries from the diagnostic biopsies of four patients with malignant melanoma, renal cancer, brain tumor (astrocytoma grade 2), and Hodgkin disease, respectively, were expressed in *E. coli* using a λZAPII phage system. To test for clones expressing antigens against which the autologous patient had specific antibodies, at least 1×10^6 recombinant clones from each tumor cDNA were screened using an ELISA modified for the exclusive detection of human IgG antibodies. Of a total of 5×10^6 clones tested, 109 were found to be reactive with IgG antibodies in the serum sample of the autologous patient at a dilution of 1:1000 (Table 1). Cross-hybridization experiments using the inserts of positive clones as probes revealed that the 109 positive clones contained 24 different inserts; thus, some inserts were expressed by multiple clones in the cDNA library of a given tumor.

Table 1. Antibody reactivity of autologous sera with recombinant clones derived from human tumor cDNA

Tumor	No. of clones tested	No. of positive clones	No. of different inserts
Malignant melanoma	1.0×10^6	40	10
Renal cell carcinoma	1.8×10^6	7	5
Astrocytoma	1.2×10^6	48	5
Hodgkin disease	1.0×10^6	14	4

Molecular Analysis of the Tumor Antigens. The inserts of positive clones were subcloned to monoclonality, and the nucleotide sequences of the cDNA inserts were determined. Seven clones have been analyzed in more detail. Two known tumor antigens were identified, two additional antigens represented known molecules for which immunogenicity in humans has not been described, and three antigens were hitherto unknown molecules. The sequences of these latter three molecules (HOM-MEL-40, HOM-RCC-3.1.3, and HOM-HD-21) have been deposited in the EMBL data base (accession nos. Z49105–7).

Melanoma antigens. The 40 positive clones from the malignant melanoma that were recognized by the patient's serum contained 10 distinct inserts. Of these, 1 each represented MAGE-1 (7) and tyrosinase (8), respectively, which had been originally defined as targets of cytotoxic T cells. In addition, 3 clones were identified as HOM-MEL-40, which represents a hitherto unknown molecule. The remaining five inserts remain to be sequenced.

Renal cell carcinoma antigens. One of the seven renal carcinoma clones coded for a new molecule (HOM-RCC-3.1.3), which at its 5′ end has 50% homology with the conserved regions and the functional domains of carbonic anhydrases (16). The sequence analysis of the four other non-cross-hybridizing inserts has not been completed.

Brain tumor antigens. Antibodies in the serum of the patient with the brain tumor reacted with 48 clones, which contained five distinct inserts. The inserts of two clones represented the *TEGT* gene. This gene is a conserved gene that is developmentally regulated in the testis (17).

Antigens expressed in Hodgkin disease. The antibodies in the Hodgkin disease patient's serum reacted with 14 clones, which appeared to contain four different inserts. Three clones represented restin, a recently described intermediate filament-associated protein. It had been shown by immunohistology that the expression of restin is restricted to Hodgkin and Reed–Sternberg cells and cultured monocytes (18). Six clones contained an insert coding for HOM-HD-21, and the two different inserts of the remaining five clones have not been sequenced. HOM-HD-21 is a new molecule that at its 5′ end shares 60% homology with an S-type lectin with a galactose-binding consensus motif (19, 20).

Expression Pattern of the New Tumor Antigens. Northern blot analysis with a variety of human tissues was performed to define the expression pattern of the newly identified tumor antigens. The melanoma antigen HOM-MEL-40 was strongly expressed in melanomas but was absent in other tissues except for normal testis (Fig. 1). The carbonic anhydrase-like protein (HOM-RCC-3.1.3) was strongly overexpressed in ≈20% of the renal cell carcinomas when compared to normal renal tissue

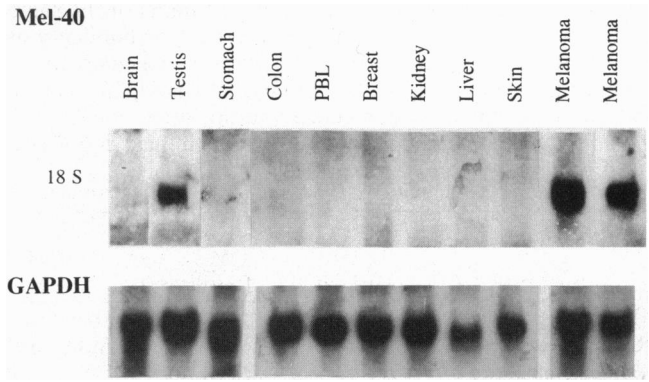

FIG. 1. Northern blot analysis of the expression of clone HOM-MEL-40, which codes for a melanoma antigen in various human tissues. PBL, peripheral blood lymphocyte.

Table 2. Expression pattern of tumor antigens in various tissues (selection)

Tissue	HOM-MEL-40*	Expression ratio HOM-RCC-3.1.3 (CAH-like)	HOM-GLIO-30.2.1 (TEGT)	HOM-HD-21 (lectin-like)
Kidney	−	1	1.5	−
Brain	−	−	1	−
Tonsil	−	−	1	1
Stomach	−	−	1.5	−
Colon mucosa	−	0.2	1.5	−
Breast	−	−	1.0	−
Skin	−	−	−	−
Testis	+	−	1	−
Renal cancer	−	>5 in 4/19 cases ≤1 in 15/19 cases	NT	−
Hodgkin disease	−	−	NT	>10
Astrocytoma	−	−	>5 in 8/12 ≤1 in 4/12	−
Melanoma	++ in 3/4 cases	−	−	−

Northern blot analysis was performed with RNA samples from tumor and normal human tissues matched by hybridization with GAPDH. Expression ratios were calculated after densitometric analysis of autoradiographs. The signal obtained with the normal counterpart of the diseased tissue was set to 1. NT, not tested; CAH, carbonic anhydrase-like.

*The expression patterns are as follows: −, no expression; +, weak expression; ++, strong expression.

(Table 2). The *TEGT* gene was overexpressed in 8 of 12 astrocytomas compared to normal human brain tissues. mRNA for the HOM-HD-21 antigen, the lectin-like structure detected by the Hodgkin patient's serum, was increased about 10-fold in diseased tissue as compared to normal tonsils. As the expression patterns of MAGE-1, tyrosinase, and restin have been investigated extensively by other groups (7, 8, 18), they were not analyzed in more detail in this study.

Occurrence of Antibodies Against Tumor Antigens. To determine the occurrence of antibodies against the newly defined tumor antigens, sera from patients with different tumors and healthy controls were tested (Table 3). While the initial screening had been performed with autologous serum diluted 1:1000, the allogeneic sera were used at a 1:200 dilution in order not to miss low-titer antibody responses. With the exception of restin, antibodies against the tumor antigens were detected at varying rates only in the sera of patients with the same type of tumor. In contrast, the rates of sera positive for antibodies against restin were similar among patients with Hodgkin disease, other tumors, and healthy controls.

DISCUSSION

To date, very few human tumor antigens that elicit an immune response in the autologous host have been defined at the molecular level. The definition of 24 such antigens in four tumors of different origin described here suggests that the analysis of B-cell responses against other tumors of the same or new types may identify a hitherto unexpected abundance of human tumor antigens. Our results with a malignant melanoma, renal cell cancer, astrocytoma, and Hodgkin disease indicate that tumor immunogenicity in the autologous host is a common phenomenon for many, if not all, human neoplasms and is not restricted to malignant melanomas. In addition, it becomes evident that the immunogenicity of human tumors is not due to the expression of a single antigen; rather, it is conferred by multiple antigens.

The observation that antibodies against the majority of these new antigens can as yet be found only in the sera of patients bearing the same type of tumor suggests that tumor growth is essential for the development of a humoral immune response against the respective tumor antigens and that antibody production is not an autoimmune effect elicited by tumor-independent mechanisms. The detection of antigens that are significantly overexpressed (e.g., the carbonic anhydrase in renal cell cancer) or even specifically expressed (e.g., the melanoma antigen HOM-MEL-40) in tumors of the same type together with the restriction of the respective antibodies to a defined patient population distinguish our study from previous attempts to define human tumor antigens by serological approaches. The most obvious difference between our and previous studies, which relied on low-titer serum antibodies or human monoclonal antibodies generated *in vitro* from patients' lymphocytes, is the stringent serological detection system, which is limited to antigens that elicit high-titer IgG responses in the patient *in vivo*. The stringent screening conditions may also increase the likelihood that the identified antigens (as suggested by sequence homologies with known molecules) may be relevant to the growth and behavior of tumor cells.

With regard to antigens that elicit antibody responses in non-tumor-bearing individuals, it is unclear why these antigens—e.g., the highly Hodgkin and Reed–Sternberg cell-specific restin (18)—are immunogenic in apparently healthy controls. One possible explanation is that the antigen may be expressed in normal cells under nonmalignant conditions such as viral infections or other inflammatory processes.

Table 3. Humoral immune responses against human tumor antigens

Subject	HOM-MEL-40	HOM-MEL-45 (tyrosinase)	HOM-MEL-55 (MAGE-1)	HOM-RCC-3.1.3 (CAH-like)	HOM-GLIO-30.2.1 (TEGT)	HOM-HD-21 (lectin-like)	HOM-HD-397 (restin)
Melanoma patients	2/11	2/11	4/11	NT	NT	NT	NT
Renal cancer patients	0/8	0/8	0/8	2/14	0/7	0/7	5/7
Astrocytoma patients	0/10	0/10	0/10	0/11	2/13	0/11	7/11
Hodgkin patients	0/10	0/10	0/10	0/17	0/17	10/18	14/17
Healthy controls	0/12	0/12	0/12	0/15	0/20	0/17	12/17

The values given are the number of subjects with an immune response/total number of subjects tested. NT, not tested.

According to our data, overexpression of antigens in the tumor compared to normal tissues seems to be the most common reason for immunogenicity. By comparing the nucleotide sequences of the antigen derived from the tumor cDNA with that derived from normal tissue, we have not detected mutations to date. Similarly, we have not detected viral sequences, even though our approach should be suitable for the detection of tumor-associated viruses. [In this regard, it should be mentioned that the neoplastic Hodgkin and Reed–Sternberg cells in the spleen from our Hodgkin disease patient were negative for Epstein–Barr virus (21)]. Whether other mechanisms are involved in the generation of an immune response to a given tumor antigen, such as *de novo* expression of antigens by the tumor that are normally expressed only during the embryonal phase or expression of xenogeneic (e.g., viral) antigens, will be answered by the analysis of additional tumors.

The initiation of a B-cell response against antigens expressed by the autologous tumor might be triggered by the presentation of the respective antigens on the cell surface of the tumor cells (this could be the case for HOM-HD-21, the lectin-like structure in Hodgkin disease) or by the exposure of proteins usually inaccessible for the immune system, which is mediated by mechanisms related to malignancy such as tumor necrosis. The latter could apply for cytoplasmic or nuclear antigens, such as restin or the TEGT protein, respectively.

The clinical significance of B-cell responses to tumor antigens is unknown. While the presence of p53 antibodies (1) is associated with a poor prognosis, the clinical significance of anti-HER-2/neu antibodies remains to be determined (2). More patients must be analyzed to determine whether the development of antibodies to tumor antigens is associated with clinically relevant features or might be used for diagnosis and prognosis. At the functional level, antibodies to intracellular proteins would not be expected to affect normal cell function; however, antibodies against surface molecules, such as the HOM-HD-21 antigen in Hodgkin disease, may interfere with its adhesive properties and influence disease progression. A functional role of this lectin-like molecule seems likely, especially in the light of the close interaction between the neoplastic Hodgkin and Reed–Sternberg cells and their surrounding T cells.

The antibodies against tumor antigens detected by our screening system were of the IgG subclass, implying that cognate helper T-cell immunity might be present and operative in patients with a respective B-cell response. Indeed, the coexistence of $CD4^+$ T cells and a B-cell response has been demonstrated for the HER-2/neu protein in patients with breast cancer (2). While much emphasis has been placed on the role of major histocompatibility complex class I-restricted $CD8^+$ T cells in the recognition of tumor-specific antigens and the effector phase of the immune response against tumors, evidence has accumulated that $CD4^+$ T cells also play a critical role in the antitumor response by mediating critical priming and effector functions. In addition, it has been shown that $CD4^+$ T-cell lines and clones cultured from tumor-infiltrating lymphocytes recognized epitopes that were products of the same tyrosinase gene, which was shown to encode class I-restricted peptides recognized by $CD8^+$ T cells (22). These results, together with our observation that tumor antigens defined by T-cell responses such as MAGE-1 and tyrosinase can also be detected by the serological approach, suggest that an integrated immune response against tumor antigens may exist that involves both $CD8^+$ and $CD4^+$ T cells as well as B cells. Since the molecular definition of tumor antigens by SEREX of tumor cDNA is fast and has a high yield, it may make sense to first define tumor antigens by SEREX and then to analyze their role in T-cell immunity. Such a strategy could provide a rapidly expanding basis for additional approaches to the immunotherapy and gene therapy of human neoplasms.

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